RESEARCH PROPOSAL

Production and Characterization of Exopolysaccharides by Bacteria isolated from effluents of Dairy Industry of Jaipur City

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Submitted by

Ishpreet Kaur

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Under the Supervision of

Dr. Charu Sharma (Supervisor)

Senior Assistant Professor

Dr.Payal Mehtani(Co-supervisor)

Associate Professor

DEPARTMENT OF ENVIRONMENTAL AND LIFE SCIENCES

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Topic:- Production and Characterization of Exopolysaccharides by Bacteria isolated from effluents of Dairy Industry of Jaipur City.

Introduction

1.1.Exopolysaccharides or extracellular polymeric subatances(EPS)

The term exopolysaccharides (EPS) was coined by Sutherland (1972) who described them as high molecular weight carbohydrate polymer formed by many marine bacteria. They are also referred as Extra Polymeric Substances (EPS) which are secreted by microorganism into their environment.Their presence can be easily detected in microbial cells by appearance of mucoid colonies (Sutherland, 1990).Microbial polysaccharides are divided into structural polysaccharides, intracellular polysaccharides and extracellular polysaccharides. The structural polysaccharides include cellulose, chitin, peptidoglycan etc which provide structural integrity to the cell. The intracellular polysaccharides occur widely in mushrooms like *Ganoderma* spp*., Inonotus* spp*., Phiebia* spp*., Pleurotus* spp. and *Inonotus* spp. They are reported to have antioxidant and anticancer activities and thus are of medicinal significance (Lee *et al.,*2007*;* Silva *et al*., 2013).Exoploysaccharides are formed by monosaccharide residues of sugar and sugar derivatives.They are produced by plants, algae, fungi and bacteria.Glucose, galactose and rhamnose in different ratios are mainly apperared in these units (Freitas *et al*., 2011). EPS producing microorganisms have also been isolated from polluted water (Fusconi and Godinho, 2002), food materials as cheese (Kojic *et al,*2015) and activated sludge(Sheng *et al.,* 2006). EPS producing microbes generally grow in a habitat that has large amounts of carbohydrate materials and small amount of nitrogenous substrates.EPS present in bacterial cells helps in cellular recognisation, aggregation and adhesion in their natural environments. It also acts as a nutrient trap, facilitating bacterial growth.Production of EPS by microorganism is advantageous because of ease of manipulation of culture conditions and cost effective recovery of product produced (Christensen *et al.,* 1990).

1.2.Applications of EPS produced by microorganisms

EPS are used in many industries as gelling agents ,flocculating agents and as emulsifiers etc.Fig (1).(Pawar *et al.*,2013).

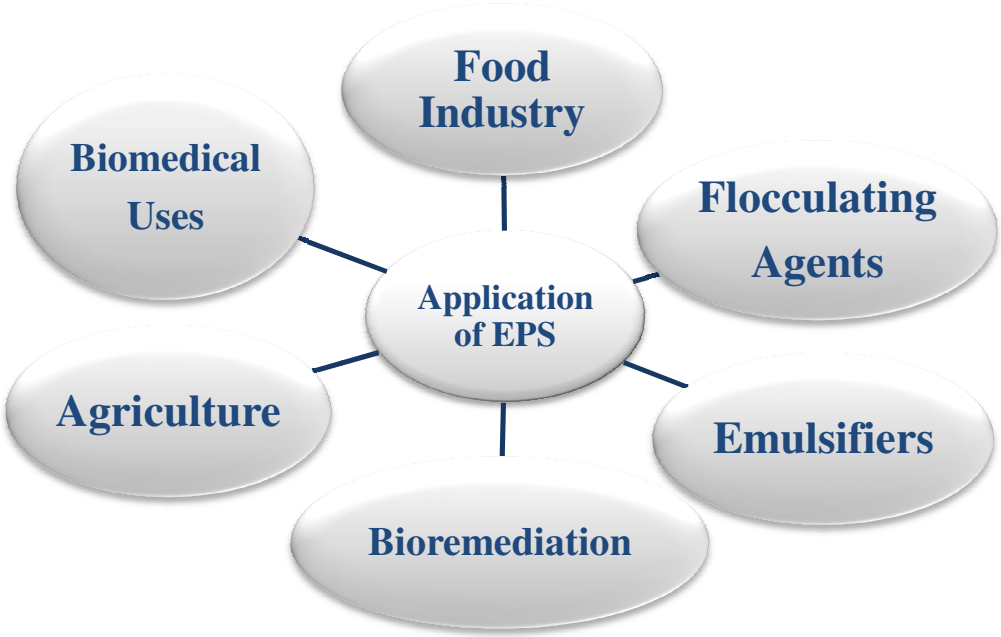

Fig.1. Applications of EPS.

1.2.1.Food Industry

Large number of polysaccharides used in food industry are of plant origin. Most of them are chemically or enzymatically modified in order to improve their rheological properties (Becker *et al.*,1998). EPS have the capacity to act as viscosifying agents, stabilizers, emulsifiers, gelling agents, or water-binding agents. These properties make them useful to be used in food and dairy industry. Dextran was the first industrial polysaccharide produced by lactic acid bacteria. It is used in confectionary to improve moisture retention, viscosity and inhibit sugar crystallization (Whistler and Daniel,1990). Curdlan from *Alacaligenes feacalis* is used as gelling agent in bakery. Gellan (PS-60 or gelrite) from *Aeromonas elodea* also forms thermoreversible agar like gels and is used as gelling agent in food and as a solidifying agent in microbiological media. Glycan from *Saccharomyces cerevisiae* is used as fat/oils substitute in low calorie diet (Sandford and Baird, 1983).

1.2.2.Flocculating Agents

Other than some use of inorganic and organic chemical compounds like aluminium sulphate, polyaluminium chloride, polymers of polyacrylamide derivatives and polyethylene imines (Zhang et al., 2007). Few studies have reported the use of EPS derived from *Virgibacillus* sp. Rob, *Bacillus* sp. Gilbert and *Arthrobacter* sp. Rates that can effectively accelerate flocculation process (Cosa *et al.*, 2011; Piyo *et al.*, 2011; Mabinya *et al.*, 2012). Use of microbial EPS as flocculating agents makes the waste water treatment process more ecofriendly and can also eliminate the adverse affects of chemicals on health.

1.2.3.Emulsifiers

EPS from microbes can form stable emulsion and can be used for oil degradation. Emulsion by *Acinetobacter calcoaceticus* RAG-1 is commercially used and was found to be more effective than other commercial gums such as arabic, tragacanth, karaya and xanthan.(Ashtapture and Shah ,1995). It is used for cleaning oil tankers and degradation of crude oil. An EPS produced by a marine bacteria, also forms stable emulsions with a number of hydrocarbons was proved to be more efficient than the commercially available emulsifiers (Iyer *et al.,* 2006).

1.2.4.Bioremediation

The potential role of EPS in the removal of heavy metals from the environment is due to their emulsification, flocculation properties and also their ability to bind metal ions from solutions. It was reported that adding EPSs produced by *Cyanobacteria* to wastewaters removes heavy metals such as copper, cadmium and lead. EPSs can physically interact with these heavy metals and take them in through biosorption(Mota *et al.,* 2016).

1.2.5 Agriculture

Exoploysaccharides can facilitate the attachment of nitrogen-fixing bacteria to plant roots and soil particles, which mediates a symbiotic relationship. This is important for colonization of roots and the rhizosphere, which is a key component of soil food webs and nutrient cycling in ecosystems. It also allows for successful invasion and infection of the host plant (Ghosh *et al.,* 2016) .

1.2.6. Biomedical Uses

A number of medical uses of EPS have been reported in literature. They have antibacterial, antiviral, antitumor and immune stimulant activities (Okutani, 1984). A low molecular weight heparin like EPS having anticoagulant property has been isolated from *Alteromonas infernus,* obtained from deep sea hydrothermal vents (Colliec *et al*., 2001). Bacterial cellulose is used for wound healing ,skin repair, and absorbent of exudates as well as permeable material. Clinical trials with bacterial cellulose dressings on acute and chronic wounds (eg.Diabetic foot ulcers) showed superior results than similar dressings from plant cellulose (Najwa *et al*., 2014).

2. Review of literature

EPS production have been observed in Bacteria (Freitas *et al.,*2009) and Cyanobacteria (Philippis *et al.,*2001) Cryptophyta (Bermudez *et al.,*2004),Mushroom (Zou *et al.,*2006),Yeast (Duan *et al.,*2008*)*and Basidiomycete (Manzoni and Rollini, 2001).EPSs of freshwater microalgae,*Scenedesmus acuminatus* (Chlorophyceae) have been explored for its metal complexing

properties (Lombardi *et al*., 2005).The polysacchardies of biological response modifiers can be isolated from bacteria, fungi, brown algae and photosynthetic plants (Leung *et al*., 2006). The common examples are *Enterobacteriaceae, Xantomonas campestris, Pseudomonas* sp and many others bacterial and fungal species (Margaritis and Pace, 1985).

Many EPS producing bacteria have been isolated from soil samples.EPS producing bacterium identified to be *Alcaligenes aqualitis GDRSGP* was isolated from soil samples that were collected from farmland at Gandervakottai, Pudukkottai (Gowsalya *et al*., 2011). Soil and water sample were taken from the different sites of Gujrat, Orissa and West Bengal. Five hundred eighty seven halophilic bacteria were isolated from the both samples of which twenty were found to be positive for EPS production. Among them the isolates SUR202, SUR307, SUR310, JW307 and JS304 were reported to give an EPS yield of 1.68-1.85g/L. Fourteen isolates were members of genera *Halomonas* and other strains belonged to genera of *Salicoccus, Bacillus, Aleromanes, Aidigimonas* and *Chromohalobacter* (Biswas and Paul, 2014). In a study thirty four EPS producing bacteria were isolated from rhizospheric soil of potato growing in Malino, South Sulawesi. These were also capable of producing Indole Acetic Acid (IAA) in range of 0.40 to 21.14mg/ml (Mu'minah *et al.,* 2015).

Another study reported that EPS producing bacteria can also be used as biofertilizer because of their soil aggregating potential. Soil samples from rhizosphere of mung bean were collected from forest area of Malang, East Java. Three bacteria SPE-2, SPE-10, and SPE-20 were observed to produce slime. These species did not show antagonistic effect to each other when cultured simultaneously and also were not pathogenic to mung beans (Arfarita *et al.,* 2016). Soil samples were collected from sugarcane field located in Gujarat region for isolation of EPS producing bacteria. Nine isolates showed the presence of mucoid colonies. EPS production was reported to be highest in isolate KPEP3 (0259 mg/ml) and KPEP4 (321mg/ml) and it also showed antibacterial activity against test isolates, *Bacillus subtilis* and *Vibrio cholera*. Biochemical characterization indicated that these isolates may belong to genera *Bacillus* (KPEP3) and *Pseudomonas* (KPEP4) (Patel *et al.,* 2018).

Biofilm forming bacteria are reported to produce EPS that contributes to their pathogenicity. Marine bacteria capable of forming biofilms, were isolated from water samples from Coastal area of Toticorin, South India and studied for EPS production. Among all isolates species B3 was highest EPS producer. Thin Layer Chromatography showed that glucose and galactose were major sugar components. IR spectroscopy revealed the presence of COOH and OH group in the EPS confirming its polysaccharide structure (Muthusamy *et al.,* 2011). Extraction of EPS was also reported from two biofilm producing bacterial isolates named as AN-6 and AN-12, which were isolated from soil sample of different areas of Gujarat (India). Results showed that EPS was anionic heteropolysaccharide with complex and fibrous

like structure. Characterization of biofilm EPS was carried out by FTIR analysis which confirmed the presence of hydroxyl, aldehyde, and alkyne groups (Parmar *et al.,* 2018).

Literature suggests that microbially produced EPS can act as better flocculating agent as compared to chemicals. Such microbial flocculants can be used for sewage treatment process. An EPS producing *Bacillus thuringiensis* 27 was isolated from sand soil in Gurban Tonggut Desert, Xinjiang, China. Maximum EPS production was achieved by orthogonal matrix method and bioflocculant activity of EPS was analyzed. EPS producing bacteria were isolated from municipal waste water treatment plant and their sludge flocculation potential was studied. The isolates produced three types of EPS as loosely, tightly and broth EPS. Flocculation activity of all isolates increased with the increase in EPS production. The zeta potential of EPS was studied and *Cloacibacterium normannese* EPS showed maximum value of -47.8 ± 0.4mV that also correlated with highest EPS production (11.8 g/l) by this isolate (Nouha *et al.,*2015). Under optimum conditions, maximum EPS production (20.19g/l) was found to be five times higher than basal conditions. The flocculation activity of the extrapolymer when used at a concentration of 0.4 mg/l was reported to be 80.4% in kaolin system suspension (Wang *et al.,* 2012).

In a study, activated sludge sample was collected from a municipal wastewater treatment plant of Bojnourd for isolation of EPS producing bacteria. Twenty isolates out of seventy four were found to produce EPS. High EPS production was observed in presence of 2.5 – 3 % glucose. The EPS concentration for all bacteria was observed to be between 72.67-139.34 g/l. Highest EPS producing bacteria were identified as members of *Bacillus, Klebsiella* and *Pseudomonas* genera on the basis of 16S rRNA gene amplification. Flocculation activity of purified EPS was measured and EPS from two isolates whose total carbon/total protein ratio was more acted as excellent flocculating agents in vitro (Shahnavaz *et al*., 2015). Not only *Phormidium* sp obtained from Microbiology department of Bharathidasan University, India also showed production of EPS. Sucrose was found to be major component of EPS by HPLC. It was reported that EPS showed good bioflocculation activity even at concentrations as low as 10mg/L. It can be directly utilized for water purification and refining processes (Baldev *et al*., 2015).

EPS produced by microbes act as excellent emulsifiers and this emulsification property is utilized in bioremediation process. Exopolysaccharide (EPS) produced by *Vibrio harveyi* strain VB23 isolated from cvoastal regions of Goa, India were studied for their emulsification activity. EPS was heteropolysaccharide in nature and had the presence of hydroxyl ,carboxylic and amides groups. The stability of emulsion formed by polymer of VB23 was observed to be comparable to other EPS producing isolates. Emulsification property of this isolate can be used in bioremediation and in oil recovery as well (Bramhachari and Dubey, 2006). Sixteen bacteria producing mucoid colonies were isolated from soil samples collected from Barnala, Punjab 16s rRNA sequencing confirmed B14 isolate to be *Alcaligenes faecalis* which was also the highest EPS producer among all of them. A production

medium supplemented with glucose and $MgSO_4$ gave the highest yield of EPS. Structural elucidation of EPS by Nuclear Magentic Resonance (NMR) revealed its tetrasaccharide nature of EPS. Fourier-transform infrared (FTIR) indicated the presence of different functional groups suggesting the heteropolymeric nature of sample. Gas Chromatography-mass spectroscopy (GCMS) analysis techniques confirmed that the presence of rhamnose, mannose and glucose in EPS. EPS showed good emulsification activity and viscosity (Kaur *et al.,*2015). *Streptococcus thermopilus* CC30 isolated from raw milk produced 1.95 g/l of EPS in skim milk lactose medium. EPS was formed of glucose and galactose (1:1 ratio). FTIR analysis showed the presence of NH_2, OH and COOH groups. EPS was found to have moderate antioxidant activity. H_2O_2 scavenging activity was found to be 71.9 ± 0.2% at a concentration of 1000 µg/ml. EPS was also capable of stabilizing emulsions with different oils and hydrocarbons (Kanamarlapudi and Muddada, 2017).Nine EPS producers were isolated from the Northern Gulf of Mexico.

EPS producing *Lactobacillus plantarum* SKT 109 was isolated from *kefir* grains collected from TIbet , China. EPS was purified and was found to be composed of fructose and glucose and had molecular weight of 2.1 x 10^6 Dalton. Milk fermentation studies showed that the strain had a long shelf life in fermented milk. When present, it increases the flavour, rheology of milk and eliminated disgusting flavour initially produced by SKT109 (Wang *et al.,* 2015).

The review of literature suggest that there are many benefits of microbially produced EPS. There is need to develop potent EPS that can be used for commercial applications like flocculation agents or flocking agents by which suspended particles are combined to form a floc so that sedimation and filteration ability improve and that can make the waste water treatment process in dairy industry more efficiently and ecofriendly .

3.Objectives:-

- Isolation of EPS producing bacteria from effluents of dairy industry
- Characterisation of chemical nature of EPS
- Application of EPS in *in-vitro* conditions

4 Methodology

4.1. Sampling site and sample collection

Samples will be collected from dairy industry in sterile bottles. They will be stored in freeze at 4°C till further processing.

4.2. Isolation and characterization of EPS producing organisms

Bacterial cultures will be isolated from water samples using standard microbiological techniques on nutrient agar media after incubation then the plates will be visually inspected for slime production. EPS producing isolates will appear slimy (Vijayabaskar , 2011). They will be studied for their morphology and Gram nature. The potent isolates showing maximum EPS production will be identified by 16S rDNA sequencing.

4.3.Optimization of physical parameters for EPS production

The physical properties ie.pH,temperature and incubation period will be optimized for maximum EPS production from the potent isolate.

4.4.Production and extraction of EPS

The isolates will be grown in broth medium .After the end of incubation period, culture will be harvested by centrifugation at 10,000 rpm for 20 min at 4°C. Culture supernatants will be used in precipitating of EPS as per the protocol given by (Kimmel *et al.,* 1998) by using ethyl alcohol. The precipitate will be collected and dried at 80°C to constant weight.

4.5.Chemical analysis of EPS

The total amount of protein and carbohydrate in EPS will be quantified using standard Bradford method (Bradford, 1976) and phenol-sulfuric acid method (Dubois *et al*., 1956) respectively.

4.6.Characterization of EPS

The morphology of EPS will be studied by SEM. For identification of sugars present in EPS chromatographic techniques will be used. Fourier transform infrared (FTIR) analysis will be carried out to identify the presence of differnt functional group such as O-H, C=O, COOH etc (Seviour *et al*., 2010)and X-Ray Diffraction will be used for identify its polymeric nature and proportion of the sample (Alexander, 1969).

4.7.*In-vitro* application of EPS

Depending upon the chemical nature of the EPS produced by the potent isolate any of the following application will be studied as Flocculation activity can be calculated by using standard kaolin suspension jar tests method to decrease the turbidity (Nouha *et al*.,2015).The work plan is summarized in Fig. 2

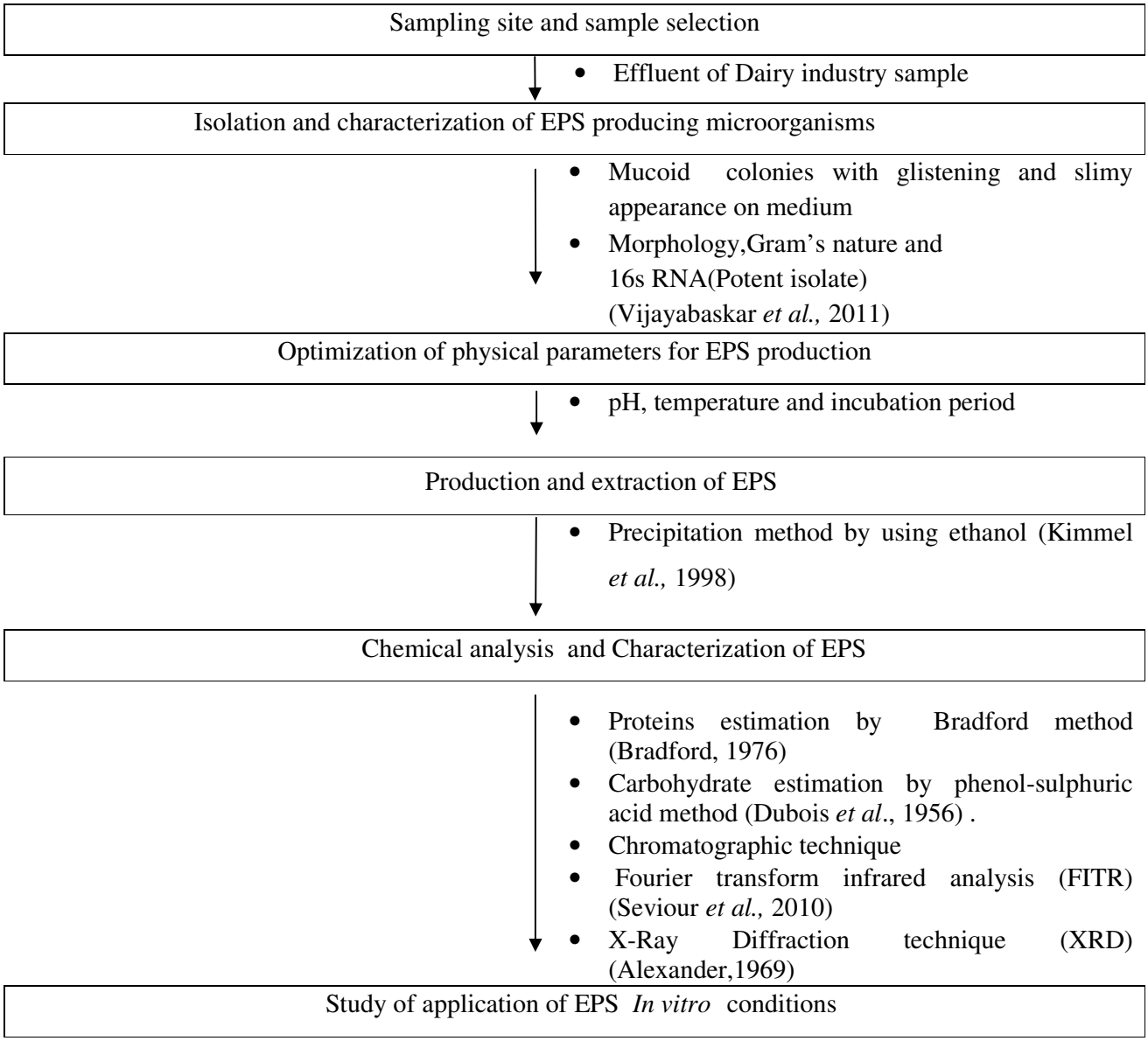
Sampling site and sample selection
• Effluent of Dairy industry sample
Isolation and characterization of EPS producing microorganisms
• Mucoid colonies with glistening and slimy appearance on medium
• Morphology,Gram's nature and 16s RNA(Potent isolate) (Vijayabaskar et al., 2011)
Optimization of physical parameters for EPS production
• pH, temperature and incubation period
Production and extraction of EPS
• Precipitation method by using ethanol (Kimmel et al., 1998)
Chemical analysis and Characterization of EPS
• Proteins estimation by Bradford method (Bradford, 1976)
• Carbohydrate estimation by phenol-sulphuric acid method (Dubois et al., 1956) .
• Chromatographic technique
• Fourier transform infrared analysis (FITR) (Seviour et al., 2010)
• X-Ray Diffraction technique (XRD) (Alexander,1969)
Study of application of EPS In vitro conditions
• Flocculation activity (Nouha et al., 2015) etc.

Fig.2 Flow chart of work plan

5.Expected Outcome

This study will be concerned with the production of extracellular polysaccharides from bacterial species isolated from dairy industry. These polysaccharides can then be tested for flocculation activity. In future, the fermentation conditions can be manipulated to enhance the production at commercial level.

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